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Molecular characterization and phylogenetic analysis of structural protein Vp1 to new isolate of duck hepatitis A virus

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ABSTRACT

Duck hepatitis A virus (DHA) is very fatal viral disease affecting young ducklings under one month old. The disease is generally spread among duckling flocks inducing sever dramatic and economic losses. The present research highlights investigation of duck hepatitis virus through collection of hundred spleen and liver field samples from various commercial 3-11 days old duckling sectors (Pekin and Mullard) at ten Egyptian governorates in 2022 and 2023 with historical view of high mortalities and nervous manifestations with background of previous immunization. The clinically infected specimens were directly screened using RT-PCR assay to detect duck hepatitis A virus through amplification of VP1 gene that reveals only one sample (obtained from Menofia governorate) was positive for DHAV-3. BLAST analysis of Partial obtained sequence of VP1 gene showed that it was closely related Egyptian strain (accession number OR543968) besides nucleotides and amino acid changes were observed in comparison with other strains. Phylogenetic analysis of the obtained strain revealed clustering with viruses of Chinese origin and distinctive from vaccinal strains utilized in Egypt. Successful isolation of duck hepatitis A virus was achieved through inoculation of tissue homogenates into allantoic cavity of 9-11 day old embryonated chicken eggs. The outcomes of this work supplied rationalized knowledge about the epidemiological criteria of DHA virus in Egypt; emphasize the significance of DHA survey and vaccine selection.

Introduction

Duck hepatitis virus is notifiable, highly contagious, acute, rapidly spreading and lethal disease up to 95% of duckling populations with substantial commercial and economic losses due to high morbidity (Liu *et al*., 2019). The disease is caused by duck hepatitis A virus (DHAV) that is grouped into three heterologous types: 1, 2 and 3. The type I DHAV (recently named as DHAV) belongs to *Picornaviridae* family within genus *Avihepatovirus* (Lefkowitz *et al*., 2018). Whereas serotypes II and III were reclassified as duck astrovirus 1 and 2 within *Astroviridae* family respectively. The type 1 (DHAV) is classified into three serotypes; serotype 1 (DHAV-1) is the most widely distributed pathogenic serotype and known as classical serotype (Kamomae *et al*., 2017). Serotype 2 (DHAV-2) recovered in Taiwan while, serotype 3 (DHAV-3) reported in China, South Korea and Vietnam (Li *et al*., 2013). No antigen relations or cross protections have been detected between the DVH three types. (Tseng and Tsai 2007).

The genome of DHA virus is non-enveloped with single stranded RNA of positive sense, about 7.7 Kb containing nearly 7800 nucleotides (Yun *et al*., 2010). The viral genome comprises single ORF encrypting about 2249 amino acids of large viral polyprotein (VP) flanked with 5′ untranslated region and 3′ with poly(A)tail (Pan *et al*., 2012). The untranslated region among DHA strains is used for diagnostic molecular purposes as it is highly conserved (Fu *et al*., 2008). During viral replication process the polyprotein (VP) is processed to three subunit or functional proteins (VP0, VP1 and VP3) in the viral capsid and other nine non-functional proteins called (2A1, 2A2, 2A3, 2B, 2C, 3A, 3B, 3C and 3D/RNA-dependent RNA-polymerase) (Kloc *et al*., 2018). These non-functional proteins are implicated in translation, transcription, viral genome replication and activity of host cells (Liu *et al*., 2022). The VP1 protein is polymorphic, highly diverse and associated with eliciting neutralizing antibodies, pathogenicity, binding of cell receptor and DHV protection (Xu *et al*., 2012). The primary epitopes of capsid proteins VP0, VP1 and VP3 enhancing the selection of vaccine (Zou *et al*., 2016). During replication of virus, 3CD protein decrease interferon regulatory factor7 expression and retinoic acid inducible factor1 to prevent non-specific antiviral immunity (Xia *et al*., 2023). Hence, translation process is initiated by the reaction between cellular like growth mRNA binding protein-1and untranslated region (Chen *et al*., 2019).

The pathognomonic picture of disease includes petechial or ecchymotic haemorrhages, necrotic, pale, and enlarged hepatitis. Swollen kidney and spleen may be noticed. Besides neurological manifestations (opisthotonus position, inversion of neck, tremors, locomtor disturbance) and sudden death (Niu *et al*., 2019). The first record of DHV was in 1945 in America (Levine and Fabricant, 1950). Then, the disease appeared in numerous countries such as Asia, Europe, South Korea, and Vietnam. The DHAV-1 was first declared in China in 1963 (Guo and Pan, 1984). The newly DHAV-3 arose in china in 2013 and it was accompanied with DHAV-1 co-infection (Wen *et al*., 2018). The discovery of DHV in Egypt was in 1970 (Shalaby *et al*., 1978). Later on, epidemiological surveillances of DHV were studied (Mahdy, 2005). Despite intensive vaccination in duck breeders' populations using live modified Rispens strain E52, DHAV-1 was recorded, and mortalities were close to 80% (Ellakany *et al*., 2002; Abd-Elhakim *et al*., 2009). Isolation and molecular characterization of DHAV from 2012 to 2014 at different Egyptian provinces revealed that the identified Egyptian viruses were closely related to DHAV-1 and clustering within Asian strains (Erfan *et al*., 2015). Investigation of DHAV using RT-PCR targets VP1 gene between 2012-2017 from backyards at some Egyptian localities at 3-21 days old ducklings with background of nervous symptoms with high mortalities demonstrated that the newly isolates were DHAV-1 and the phylogenetic analysis showed clustering of these isolates into genogroup 4 with high homology to other Egyptian strains (Hisham *et al*., 2020). Recently, whole genome sequencing and phylogenetic tree of five DHV isolates based on VP1 gene of three to eleven days old duckling obtained from six Egyptian governorates indicated that newly DHAV strains

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were classified as DHAV-1. The viruses were categorized to four divergent groups and clustered within group 1 (Rohaim *et al*., 2021). DHAV-3 was first reported in Egypt between 2016 and 2018 in Sharkia governorates. The newly five obtained strains clustered separately, unclose to DHAV-1 Egyptian vaccine and possessed three inimitable amino acid substitutes (Hassan *et al*., 2020).

Recurrent outbreaks were recorded by DHAV-3 in Egyptian duckling flocks (Yehia *et al*., 2021). Co-infection of DHAV with other viruses as avian influenza (Mansour *et al*., 2018) and egg drop syndrome have been identified (Zhang *et al*., 2018). Therefore, DHAV control represents a great challenge. Traditional diagnostic tools depend on symptoms, macroscopic lesions with assurance of isolation and serological techniques. However, these tools are less sensitive and exert efforts so development of RT-PCR aids in detection and characterization of DHAV (Wen *et al*., 2014). Thus, fast diagnosis and prevention are the most efficient approaches for the disease control (Woolcock, 2003). So, the current study aimed to investigate the prevalence of the current DHAV circulating in Egypt.

Materials and methods

Ethical approval

All procedures of this research were carried out based on the protocols and recommendations of the ethical Committee of Veterinary Vaccine and Serum Research Institute, Agriculture Research Center, Cairo, Egypt.

Sampling (collection and handling), area of study and period

During the period from 2022 to 2023, fifty field samples (spleen, liver, and kidney) were collected from freshly died or euthanized duckling (4-15 days old) at different commercial farms (Mulland and Pekin) that belong to ten Egyptian governorates (Menofia, Giza, Qaluibya, Sharkia, Behera, Fayoum, Gharbyia, Ismailia, Demiatta and Beni-Seuf) (Table 1). Affected ducklings suffered from depression, nervous manifestations with 50-70% mortality level within five days. Macroscopic examination of hepatic lesions showed haemorrhage, swelling and enlargement. Spleen is spotted and distended while kidneys were swollen with congested renal blood vessels congestion. The examined specimens from each governorate were pooled per farm, examined as one specimen per farm and stored at -80◦C until use.

Using tissue lyser, (Tissue lyser LT compact bead mill, Qiagen) the tested organs were undergone homogenization using sterile PBS (W/V) to form 10% suspension followed by freezing and thawing for three successive cycles. Finally, centrifugation was applied at 12000 rpm for 10 minutes to separate the supernatant which was stored at -80°C till use (Woolock, 1998).

RT-PCR and virus isolation

The supernatants of homogenized tissue were subjected to extraction of viral RNA by QIAamp Viral RNA Mini kit (Qiagen, Germany, GmbH) based on the constructor's instructions. Concisely, incubation of 140 µl of suspension specimen with 560 µl of AVL lysis buffer and 5.6 µl of carrier RNA was performed for ten minutes at room temperature. After that 560 µl of 100% ethanol to lysate was added. Then, centrifugation and washing of sample was carried out based on manufacturer's protocols. Finally, elution of nucleic acid with 60 µl elution buffer provided in the kit was implemented.

Oligo-nucleotide primers (Metabion) was used in RT-PCR assay (Table 2) for the detection of DHAV through partial amplification of 5 UTRL (Fu *et al*., 2008), VP1 gene of DHAV-1 (Mansour *et al*., 2019) and DHAV-3 (Doan *et al*., 2017). The PCR amplification was applied through primer utilization in 25 µl reaction containing 12.5 µl of Quantitect probe rt-PCR buffer (QIAgen, Gmbh), 1 µl of each primer of 20 pmol concentration, 0.25 µl of RT-enzyme 5.25 µl of water, and 5 µl of template.

Table 1. Samples data of tested DHAV surveillance in 2022 and 2023.

Lab. code	Governorate	Number of samples	Year	Breed	Age
1	Menofia	10	2023	Pekin	10 days
2	Giza	10	2023	Pekin	6 days
3	Qaluibya	10	2022	Pekin	7 days
4	Sharkia	10	2022	Pekin	9 days
5	Behera	10	2023	Pekin	4 days
6	Fayoum	10	2023	Muscovy	7 days
7	Gharbyia	10	2022	Muscovy	5 days
8	Ismailia	10	2022	Muscovy	8 days
9	Demiatta	10	2023	Pekin	7 days
10	Beni-Seuf	10	2023	Muscovy	6 days

The reaction of PCR for 5 UTRL involved reverse transcriptase for one hour at 42°C. The steps of cycling reaction were three minutes at 94°C (initial denaturation) followed by thirty seven cycles of 30 seconds at 94°C (denaturation), 30 seconds at 50°C (annealing) and 45 seconds at 72°C (extension) with final extension at 72°C for 10 minutes.

PCR reaction of DHAV-3 was 50°C for 30 minutes for reverse transcription, a primary denaturation at 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds (denaturation), 55°C for 40 seconds (annealing) and 72°C for 45 seconds (extension). A final extension step was done at 72°C for 10 minutes. Meanwhile, PCR protocol of DHAV-1 were 30 minutes at 50°C for reverse transcription, 15 minutes at 94°C for activation of PCR, then, 35 cycle of 1 minute at 94°C for denaturation, 1minute for 52°C for annealing of primers, and 1 minute 72°C for extension. The last extension was implemented for 10 minutes at 72°C. The separation of PCR products was applied on 1.5% agarose gel (Applichem, Germany, GmbH) by electrophoresis in 1x TBE buffer at room temperature using gradients of 5V/cm. For analysis of gel, 15 µl of the PCR products was loaded in each slot of gel using 100 bp A gene ruler DNA ladder (Fermentas, Germany) to determine the sizes of fragment. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and data analyzing was applied using computer software (Automatic Image Capture Software, Protein Simple formerly Cell Biosciences, USA).

Isolation of virus was carried out from RT-PCR positive specimens by inoculation of tissue homogenate supernatant in 8-10 days old embryonated chicken eggs via allantoic route for three successive passages and incubation of inoculated eggs at 37°C for 7 days. Embryos were inspected

Table 2. Oligonucleotide forward (F) and reverse (R) primer sequences specific to 5'UTR and DHAV VP1 genes.

Gene	Primer sequence	Product size	Reference
UTR	F. primer CCTCAGGAACTAGTCTGGA R. primer GGAGGTGGTGCTGAAA	250 bp	Fu *et al*. (2008)
DHAV-1	F. primer ACACCTGTTTGGGAGGCAAT R. primer TCCAGATTGAGTTCAAATGCTAGTG	609 bp	Mansour *et al*. (2019)
DHAV-3	F. primer ATGCGAGTTGGTAAGGATTTTCAG R. primer GATCCTGATTTACCAACAACCAT	880 bp	Doan *et al*. (2016)

for pathological or macroscopic lesions. The collected allantoic fluid was tested for Haemagglutinating viruses (OIE, 2017).

Sequence of the PCR products and phylogenetic analysis

For partial sequences of DHAV VP1 gene, PCR products were purified by QIAquick PCR Purification Kit (Qiagen) based on the manufacturer's protocols using forward primers. The sequence reaction was applied using BigDye™ Terminator cycle sequencing kit followed by purification using Invitrogen™ Centri- Sep™ Spin Columns. Sequences of DNA were achieved using Applied Biosystems 3130 genetic analyzer (HITACHI, Japan) in Elim Biopharmaceuticals Inc., CA, USA. BLAST analysis (Altschul *et al*., 1990) was primary used to create identity of VP1 gene sequence to accession numbers of GenBank to the retrieved database comprising all DHAV strains related to genotypes1and3. Using BioEdit program Version 7.0.5.3 (Hall, 1999), the sequences of amino acids were deduced from the created nucleotides for multiple alignments and sequence identity matrix were calculated after trimming of sequences.

Phylogenetic tree was constructed by neighbor joining method using MEGA X program (Kumar *et al*., 2018) with bootstrap 1000 replicates based on VP1 gene nucleotide sequence representative strains.

Results

Results of RT-PCR and virus isolation

According to UTR, out of ten tested samples, three samples (30%) (obtained from Menofia, Giza and Qaluibya) were positive for DHAV with product seize of 250 bp (expected site) whereas the remaining samples were negative of DHAV. Based on VP1 gene amplification, the three positive viruses of DHAV were subsequent identified by RT-PCR which revealed that only one virus related to DHAV-3 with 880bp (expected site) amplicon size and negative amplification of DHAV-1 (Fig.1). Also, the positive control strain of DHAV-3 (designated as *Avihepatovirus* A isolate 101 with accession number MK862185) produces the same amplicon at 880bp meanwhile, the positive control strain of DHAV-1(designated as *Avihepatovirus* A isolate DVH-Dak-Pk-F36-2022 with accession number OP374129) did not produce any product.

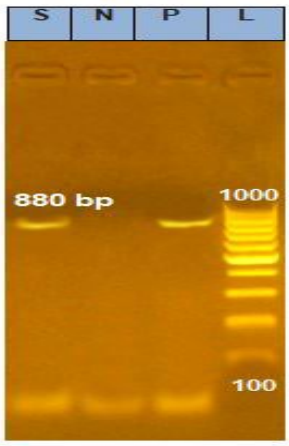

Fig. 1. PCR products of 1.5% gel electrophoresis based on both DHAV-1(609bp) and DHAV-3 (880bp) to the three strains obtained from Menofia, Giza and Qaluibya governorates. The size of amplicon was observed at 880 bp only indicating the presence of DHAV-3 only. (S)= sample, (N) = nuclease free water as negative control, (P) = (DHAV-3 strain designated as *Avihepatovirus* A isolate 101 with accession number MK862185) as Positive control.

Inoculation of the three DHAV viruses (based on RT-PCR) into allantoic cavity of 8-11 day old embryonated chicken eggs for three successive times revealed embryonic deaths in inoculated eggs after 7 days for all DHAV. The dead embryos show edema, stunting, haemorraghic enlarged liver. Kidneys were congested and spleens were mottled and enlarged (Fig. 2). The allantoic fluid collected from inoculated eggs was negative for HA viruses as NDV, A.I, EDS and duck adenovirus-1that prevalent in Egypt.

Fig. 2. isolation of DHAV on specific pathogen free eggs showing embryonic deaths seven days post inoculation with characteristic DHAV pathogenic lesions. (A)= inoculated embryos with DHAV. (B)= negative control embryos.

Results of sequencing analysis and phylogenetic tree of DHAV-3

BLAST analysis of DHAV-3 based on partial VP1 gene sequence showed that the novel strain was closely related to Egyptian strain (designated as DHAV-3 isolate DVHAYAEG1 with accession number OR543968). The newly obtained sequence was submitted to gene bank and had new accession number (PP889387).

Multiple sequence nucleotide and amino acid alignment of newly obtained sequence (DHAV-3) with some Egyptian and global strains of DHAV-3 and DHAV-1 revealed several substantiations at amino acid and nucleotides levels (Figs. 3 and 4).

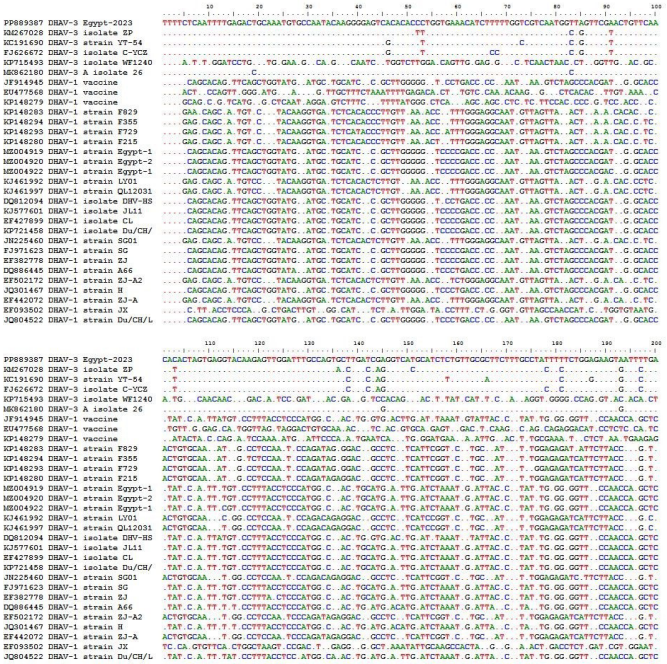

Fig. 3. Multiple alignment nucleotide sequence of the obtained DHAV-3 compared to various known global strains, constructed by BioEdit version 7.0.5.3., similarities presented as dots whereas differences represented as letters.

Sequence identity matrix of new DHAV-3 strain (PP889387) showed high identity percent to DHAV-3 strains originated from China with high similarity to Egyptian strain (accession number MK862180). The new strain of DHAV-3 showed low identity to vaccinal strains and DHAV-1 (Fig. 5).

Phylogenetic tree of VP1 gene showed that the newly DHAV-3 strain (accession number PP889387) was clustered within the strains of Chinese origin and clustered within Egyptian strain (accession number OR543968) (Fig. 6).

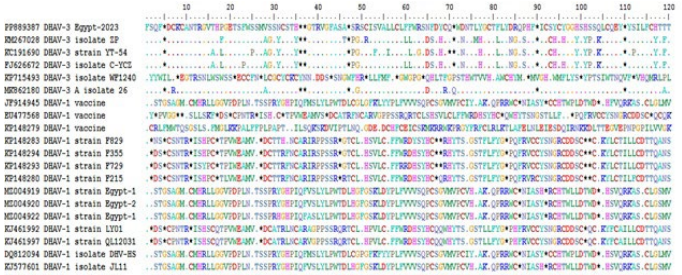

Fig. 4. Multiple alignment amino acid sequence of the obtained DHAV-3 compared to various known global strains, constructed by BioEdit version 7.0.5.3., similarities presented as dots whereas differences represented as letters.

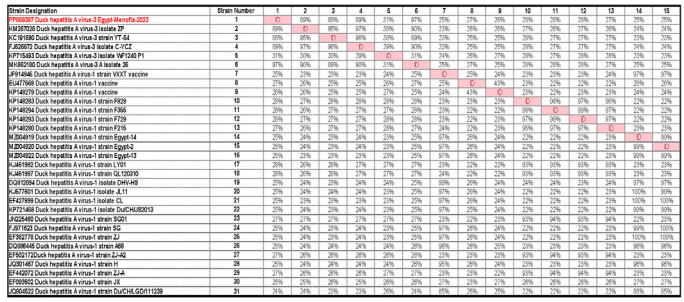

Fig. 5. Sequence identity matrices constructed by BioEdit program version 7.0.5.3. of newly obtained strain (accession number PP889387) in comparison with other representative strains of DHAV showing the identity percent.

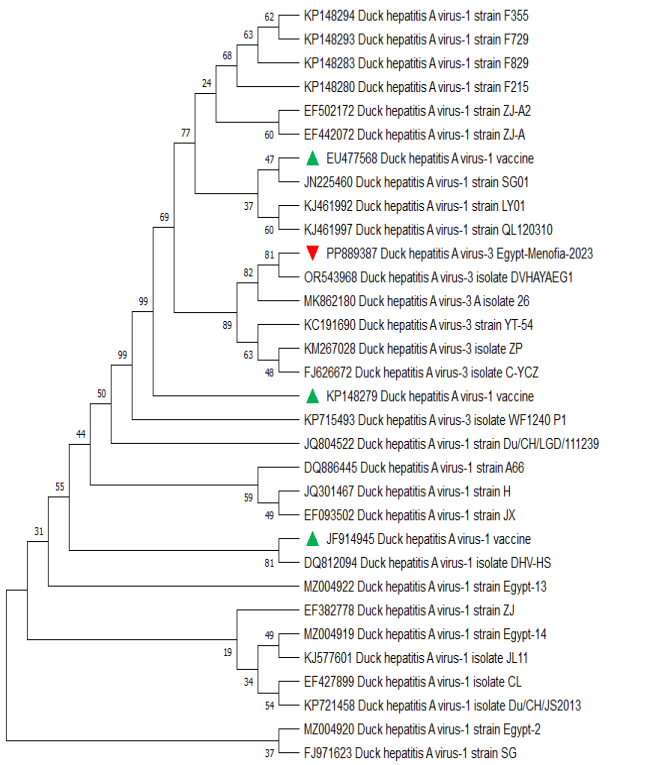

Fig. 6. phylogenetic analysis of DHAV-3 VP1 gene (red triangle color) to different representative DHAV-3, DHAV-1 and vaccinal strains (green triangle color) strains generated by MEGA X using neighbor joining method following 1000 replicate bootstrapping showing clustering of new DAHA-3 within strains of Chinese origin.

Discussion

Since 1950, both novel duck hepatotrophic and standard strains have been recognized to induce the disease that historically termed DHV (Yang *et al*., 2021). DHAV causes destructive fatalities in duck production owing to high mortality levels that might be surpasses up to 95% under field situations moreover, the ill developed immune system of duckling exaggerates the clinical disease and incapable of inducing protection from infection of virus and replication (Song *et al*., 2014). Also, vertical and horizontal DHAV transmission with virus dissemination is another necessary factor of infection (Zhang *et al*., 2021). As all infectious diseases, prevention and control policies rely on mainly strict hygienic measures and vaccination strategies besides, disease and epidemiological mapping of different viral strains which aids in diagnosis of DHAV to detect emerging and re-emerging serotypes of field virus because vaccination is serotype dependent without cross protection (Zhang *et al*., 2023).

The typical form of DHAV infection and pathological lesions are useful in primary diagnosis. In this respect the current work was designed to investigate genetic characterization of DHAV prevalent in Egypt through collection of hundred field samples from different Egyptian governorates (Table 1) with historical picture of high duckling deaths and nervous manifestations. The affected duck populations had mortality percent ranged from 50-70% within four to five days and these outcomes agree with records of DHAV infection (El-Kholy *et al*., 2021). These samples were pooled and directly screened with RT-PCR which is considered the most accurate and rapid tool for diagnosis of DHAV as reported by OIE (2018). In this study, RT-PCR was initially used for targeting UTR gene to all ten collected field samples revealing only three samples were positive DHAV. Similarly, RT-PCR was used to target the same gene (Fu *et al*., 2008). Also, UTR RT-PCR was used to detect DHAV demonstrating ten samples were positive out of thirty infected samples obtained from different Egyptian governorates as reported by Hisham *et al*., (2020). Lately, RT-PCR based UTR was used for screening clinically infected ducklings showing twenty nine positive samples out of thirty eight screened samples collected from some Egyptian governorates (Lelwa *et al*., 2023).

The VP1 protein is the outer protein that induces neutralizing antibodies via reaction with cell receptors. In addition,, analysis of VP1 gene is the main tool for DHAV characterization, genotypic mapping or serotyping and further vaccine development (Ma *et al*., 2015). Hence, the three positive UTR RT-PCR samples obtained in this work were undergone consequent genotyping identification targeting VP1 gene using another RT-PCR cycle revealing only one positive DHAV-3 (Fig. 1). In accordance with this study, out of fifteen isolates, five DHAV-3 isolates were identified as DHAV-3 directing VP1 amplified genotype-3 (Hassan *et al*., 2020). Parallel with this study; two isolates out of nine were further identified as DHAV-3 depending on amplification of VP1 gene (El-Kholy *et al*., 2021). For definitive diagnosis, the three DHAV positive RT-PCR samples were used for isolation of virus demonstrating deaths of embryos after seven days incubation period indicating the success of virus isolation (Fig. 2) with pathognomonic lesions including edema, necrosis of liver, haemorrhage, and dwarfing. In the same way, DHAV isolation was achieved after 7 days post inoculation with 80% embryonic deaths (Mansour *et al*., 2019). Successful isolation of three DHAV strains was attained using embryonated duck embryos accompanied with mortalities at the end of incubation period (Li *et al*., 2013; Zhang *et al*., 2021; Rohaim *et al*., 2021). On the contrary, embryonic deaths were observed within two to four days post inoculation (Jin *et al*., 2008). Also, the virus was only recovered from 29.4% of clinically infected samples might be maternally derived antibody interference in embryonated duck eggs (Erfan *et al*., 2015).

Sequence analysis of new DHAV-3 strain (accession number PP889387) showed numerous substitutions nucleotide and amino acid at several loci in comparison with some representative DHAV-3 and DHAV-1 indicating the presence of mutations at new strain (Figs. 3 and 4). Sequence identity matrix of new DHAV-3(PP889387) showed 89% identity to DHAV-3 strains of Chinese origin (accession numbers KM267028, FJ626672), 88% identify to DHAV-3 strain of Chinese origin (accession number KC191690), 31% identity to DHAV-3 strain of Chinese origin (accession number KP715493), whereas it had 97% identity to Egyptian strain accession number MK862180). Identity percent variations owing to adaptation of host that result from genomic divergence of the same genotype (Kim *et al*., 2008). The newly identified strain showed 25%, 27% and 26% identity to vaccinal strains with accession numbers JF914945, EU477568

and KP148279, respectively and nearly the same percentages with DHAV-1 (Fig. 5).Likewise, molecular analysis of five DHAV strain showed the close relationship to DHAV-3 Egyptian genotype with high identity percentage 88%-100% with low similarity to vaccinal strains (El-kholy *et al*., 2021; Lelwa *et al*., 2023) indicating the endemic status and establishment of DHAV-3 (Hassan *et al*., 2020; Yehia *et al*., 2021). Clustering of newly obtained strain within Chinese origin strains (Fig. 6) using phylogenetic tree revealing the origin of this strain. Also, the novel strain was distinctive from vaccinal strain used in Egypt and this agrees with (Zanaty *et al*., 2017). The diversity between the circulating DHAV-3 and vaccinal strains is potential for breaks arising. The currently used vaccine (modified live E52 Respiens) in Egypt was incapable of attaining protection against heterologous virus which may result in vaccine failure. Moreover, no records were present concerning the immunization state of duck breeders' flocks and it is unclear whether the recurrence of DHAV epidemics owing to low maternal antibody titers, genetic diversity or virulence origin might be out of capsid viral protein (Wang *et al*., 2008). The divergence between the vaccinal and field DHAV is attributed to accumulation of point mutations or genetic recombination between DHAV strains (Wei *et al*., 2012). Also, these mutations are implicated in DHAV strains evolution and consequently new strains are emerging leading to diagnosis confliction (Feher *et al*., 2021). So, updating DHAV vaccines is highly needed based on recent prevalent strains recorded from duckling infection or breaks (Li *et al*., 2013). Furthermore, full genome sequencing supplies substantial knowledge which will show the pathogenic virus pathway serving in updating effective vaccines against infection (OIE, 2010).

Conclusion

Overall, comparative studies on molecular and antigenic relations are needed to identify different DHAV based on pathogenicity pointers. Meanwhile in vivo studying is not only required for assessment of vaccine efficiency and detection of newly emerging strains but also to overcome immunization failure and following progression alterations. The genetic alterations between the latest Egyptian viruses and utilized vaccines are considered fundamental tool for vaccine effectiveness and development of novel vaccines.

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Conflict of interest

The authors declare that they have no conflict of interest.

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